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The Genetics of Polyamine Synthesis in *Neurospora crassa*[1]

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New mutations of the polyamine pathway of *Neurospora crassa* fell into three categories. The majority affected ornithine decarboxylase and lay at the previously defined *spe-1* locus. One mutation, JP100, defining the new *spe-2* locus, eliminated *S*-adenosylmethionine decarboxylase and led to putrescine accumulation. Revertants of this mutation suggested that the locus encodes the enzyme. Two other mutations, LV105 and JP120, defined a third locus, *spe-3*. Strains with these mutations also accumulated putrescine and were presumed to lack spermidine synthase activity, which catalyzes the formation of spermidine from putrescine and decarboxylated *S*-adenosylmethionine. The three *spe* loci lay within about 20 map units of one another on the right arm of Linkage Group V in the order: centromere–*spe-2*–*spe-1*–*spe-3*. The requirement for spermidine for growth was much less in *spe-2* and *spe-3* mutants than in *spe-1* mutants, which do not accumulate putrescine. This suggested that putrescine fulfills many, but not all, of the functions of spermidine, or that high levels of putrescine render spermidine more effective in its essential roles. © 1990 Academic Press, Inc.

Polyamines (putrescine, spermidine, and spermine) are essential for normal growth of all organisms (1, 2). Polyamines bind to nucleic acids and facilitate protein synthesis, but their precise roles *in vivo* are still unclear (1). The activities of polyamine biosynthetic enzymes are elevated at the onset of growth, in rapidly growing cells, and in cells deprived of polyamines (3, 4). Two decarboxylases initiate the polyamine biosynthetic pathway (Fig. 1). The first, ornithine decarboxylase (ODC),[4] converts ornithine to putrescine. The second decarboxylase, *S*-adenosyl methionine decarboxylase (SDC), converts *S*-adenosylmethionine (SAM) to decarboxylated SAM (dcSAM). dcSAM donates its aminopropyl groups in the conversion of putrescine to spermidine, catalyzed by spermidine synthase, and in the conversion of spermidine to spermine, catalyzed by spermine synthase.

The fungus *Neurospora crassa* has a simple polyamine biosynthetic pathway, in which little spermine forms (5). The *spe-1* gene of *N. crassa* codes for ODC (6); genes for *S*-adenosylmethionine decarboxylase and spermidine synthase have not been identified. In this report, we genetically map and characterize three new polyamine biosynthetic mutants of *N. crassa* at two new loci, *spe-2* and *spe-3*. The *spe-2* locus encodes SDC; *spe-3* presumably specifies spermidine synthase. The relationship of polyamine pools to growth rate shows that spermidine is essential for growth even in the presence of excess putrescine.

MATERIALS AND METHODS

Strains and growth. The strains used for crosses, growth, and biochemical analyses are listed in Table I. The medium used for all growth and biochemical analysis was Vogel's medium with or without spermidine (7). Liquid media were inoculated with conidia harvested from cultures grown on solid Vogel's medium containing 2 mM spermidine. Liquid cultures, when supplemented, contained 1 mM spermidine. Inositol (50 mg/liter) was added to cultures of *inl*-containing strains. Arginine (1 mM) was used to feedback-inhibit ornithine synthesis. Standard genetic techniques were used (8), using media with 2 mM spermidine for ascospore germination.

Liquid cultures were of two sorts. Stationary cultures were grown in 25 ml Erlenmeyer flasks with 10 ml media inoculated with two drops of a light conidial suspension. The cultures were grown for 48–60 h

[4] Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); SDC, S-adenosylmethionine decarboxylase (EC 4.1.1.50); ARG, arginine; SAM, S-adenosylmethionine; dcSAM, decarboxylated S-adenosylmethionine; PUT, putrescine; SPD, spermidine; SPM, spermine.
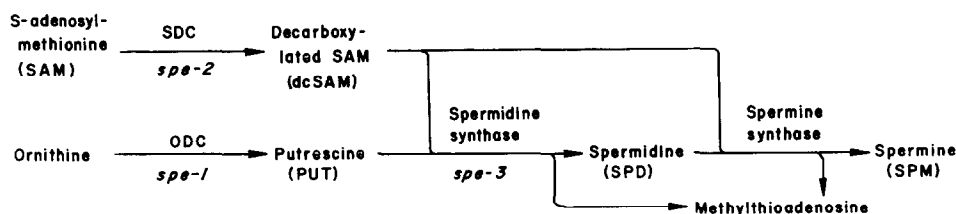
FIG. 1. The polyamine pathway of *Neurospora*, showing the positions of the metabolic blocks described in this paper.

at 32°C. Mycelial pads were harvested and dried for 2–3 h at 80°C. Exponential cultures were grown in 250- to 1000-ml boiling flasks and heavily aerated (8). Such cultures were used to determine exponential growth rates, polyamine pools, and enzyme activities. They were heavily inoculated (about 2 × 10⁶ conidia per milliliter). Polyamine auxotrophs that do not grow measurably in minimal medium in stationary culture grow well initially (depending on the mutant) in the more heavily inoculated exponential cultures owing to carryover of spermidine in the inoculum.

Mutagenesis and genetic analyses. The *aga inl* strain yielded the JP100 mutation after ultraviolet mutagenesis in inositolless death enrichment as previously described (9). The enrichment medium was modified by use of 10 mM cyclohexylamine, an inhibitor of spermidine synthase, to curb the growth of partial mutants. The LV105 mutation was selected from the *aga inl* strain by P. E. Eversole-Cire in previous work (9). The JP120 mutation, selected from the *spe-1 aga inl* strain, was separated from the *spe-1* mutant by outcrossing before analysis.

New spermidine auxotrophs were tested on agar medium for their polyamine requirements. Crosses were done by standard methods. Analyses of crosses involving the JP120 allele, too leaky for effective growth tests, were done by thin-layer chromatography as indicated below. Complementation tests were done in 1 ml medium in 13 × 100-mm culture tubes (8). Revertants of a *spe-2, inl* strain were selected on medium lacking spermidine; back- and outcrosses were used to test the allelism of the reversion event with the *spe-2* mutation, as well as its linkage to *inl*.

Polyamine determinations. Polyamine pools of exponential cultures were analyzed by high-performance liquid chromatography as

reported previously (10). Cells, collected by filtration, were washed with 0.25 M NaCl before extraction for polyamine determinations to eliminate bound, extracellular polyamines.

Qualitative analysis of polyamine pools of the progeny of JP120 crosses was done by thin-layer chromatography of progeny extracts. The progeny were grown in 3 ml liquid Vogel's minimal medium in 18 × 150-mm culture tubes for 2–3 days at 32°C. Mycelia were harvested on a 5-μm filter and washed with water and 0.25 M NaCl. The pads were extracted in 200 μl of 0.4 M perchloric acid with 1 mM EDTA at room temperature. Supernatants were dansylated by addition of 150 μl dansyl chloride (5 mg/ml in acetone) and 20 mg of solid sodium carbonate. After at least 2 h dansylated material was extracted in 250 μl ethyl acetate; the extracts were evaporated and reconstituted with ethyl acetate in volumes (15–100 μl) roughly proportional to the size of the mycelial pad. This standardized the amount of cellular material represented in standard volumes on Sil G thin-layer plates, which after development (11) could be evaluated directly by eye.

Enzyme extraction and determinations. Extracts were made at 4°C from mycelia harvested by filtration from aliquots (250–1000 ml) of exponential cultures. Moist pads were sand ground in a buffer containing 50 mM potassium phosphate (pH 7.3), 2 mM dithiothreitol, 1 mM magnesium chloride, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (12). The crude extract was centrifuged at 12,500g for 15 min, and the supernatant was desalted on a Sephadex G-25 column. Crude extracts were assayed immediately or stored at −70°C. Protein was determined by the Bradford method (13).

ODC assays were performed as described previously (14). SDC assays were performed according to Stevens *et al.*, with minor variations (12). The SDC assay buffer contained 100 mM *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid, pH 7.4, 0.20 mM unlabeled SAM, 1 mM putrescine, and 0.025 μCi of *S*-adenosyl-L-[carboxyl-¹⁴C]methionine in a final volume of 0.3 ml.

Materials. Most biochemicals were purchased from Sigma Chemical Company. Labeled SAM and [1-¹⁴C]ornithine was purchased from Amersham. Unlabeled SAM was purified on a carboxymethyl cellulose column before use in SDC assays by the procedure of Chirpich (15). SAM (20–30 mg) in 2 ml 5 mM sodium acetate buffer (pH 5.0) was loaded onto the column. The column was washed with 5 mM sodium acetate, and the eluate was monitored at 260 nm. After the first peak was fully eluted by sodium acetate, 40 mM HCl was used to elute SAM, which was stored after it emerged at −70°C. The purity of the SAM was verified by high-performance chromatography on a Partisil SCX silica cation-exchange column (16).

RESULTS

Mutant Selection and Mapping

Spermidine auxotrophs were selected as described under Materials and Methods from uv-irradiated, polyamine-starved conidia. The majority of spermidine auxotrophs found were *spe-1* mutants: they could use pu-

TABLE I

Strains Used in This Paper

Strain	Mutant loci	Alleles	Source
IC-3	*aga*	UM906	R. Davis
IC-680	*his-1*	Y175M650	FGSC[a]
IC-602	*inl*	89601	FGSC
IC-40	*aga, inl*	UM906, 89601	R. Davis
IC-1894-53	*spe-1, aga*	LV10, UM906	R. Davis
IC-2568-15	*spe-2, aga*	JP100, UM906	This paper
IC-2702-16	*spe-2, aga*	JPR2, UM906	This paper
IC-1898-61	*spe-3, aga*	LV105, UM906	P. E. Eversole
IC-2552-5	*spe-3, aga*	JP120, UM906	This paper
IC-2669-185	*spe-3, his-1*	LV105, Y155M302	This paper
IC-1894-27	*spe-1, aga, inl*	LV10, UM906, 89601	R. Davis
IC-2551-69	*spe-2, aga, inl*	JP100, UM906, 89601	This paper
IC-2551-84	*spe-2, aga, inl*	JP100, UM906, 89601	This paper
IC-2687-4	*spe-2, aga, inl*	JPR1, UM906, 89601	This paper

[a] FGSC, Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66103.

TABLE II

Three-Point Crosses Involving *spe-2* (JP100)
and *spe-3* (LV105)

Cross: *spe⁺ his-1 inl⁺* × JP100 *his-1⁺ inl*		Cross: *his-1 inl⁺* LV105 × *his-1⁺ inl spe⁺*	
Progeny genotypes[a]	No.	Progeny genotypes[a]	No.
spe⁺ his-1 inl⁺	129	*his-1 inl⁺* LV105	62
JP100 *his-1⁺ inl*	18	*his-1⁺ inl spe⁺*	83
spe⁺ his-1⁺ inl	10	*his-1 inl spe⁺*	11
JP100 *his-1 inl⁺*	1	*his-1⁺ inl⁺* LV105	7
spe⁺ his-1 inl	8	*his-1 inl⁺ spe⁺*	3
JP100 *his-1⁺ inl⁺*	1	*his-1⁺ inl* LV105	3
JP100 *his-1 inl*	0	*his-1 inl* LV105	0
spe⁺ his-1⁺ inl⁺	0	*his-1⁺ inl⁺ spe⁺*	1

| Map: *spe-2–his-1–inl* | | Map: *his-1–inl–spe-3* | |
| cM[b]: | 6.6 5.4 | cM: | 11.2 4.1 |

[a] Genotypes are given using the correct gene order; in successive pairs, they are parental, recombinants for the first interval, recombinants for the second interval, and recombinants for both intervals (double crossovers). The difference in the two crosses of map distance for the *his-1–inl* interval is large, but not unusual for this organism.

[b] cM, map unit (1% recombination).

trescine for growth, and they failed to complement other *spe-1* mutants. Only three mutations (LV105, JP100, and JP120) unable to use putrescine to satisfy their polyamine requirement appeared. All were "leaky"; that is, able to grow to some extent in minimal medium. When grown in minimal medium, they accumulated putrescine and had low spermidine pools (see below). JP100 and LV105 complemented *spe-1* mutants and one another. This implies that JP100 and LV105 affect two different loci, encoding the two main enzymes not previously represented by mutation. JP120 was too leaky to test by complementation.

Allelism of JP120 was tested in intercrosses. To distinguish the phenotype of JP120 progeny from that of wild type, extracts of minicultures were subjected to thin-layer chromatography, in which putrescine accumulation by the mutant is obvious. Among progeny of a cross involving mutations JP120 and JP100, 10.5% were wild type. No wild types appeared in the cross involving mutations JP120 and LV105. We conclude that JP120 and LV105 are allelic, and that JP100 represents another locus.

The two new loci, named *spe-2* and *spe-3*, were mapped. The strains used in mutant selection all contained the *inl* mutation, on the right arm of Linkage Group V. Outcrosses of the new mutants revealed that both new loci, like the *spe-1* locus, are linked to *inl*. Three-point crosses, using *inl* and *his-1* (Table II), and intercrosses, established that all *spe* genes lie within 21 map units of each other, interspersed with other loci (Fig. 2). This interval is approximately 2% of the genetic
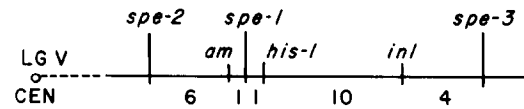

FIG. 2. Partial genetic map of the right arm of Linkage Group V, with approximate map distances based on crosses in the text and others done in connection with this work. Gene symbols: *am*, amination-deficient; *his-l*, histidine requiring; *inl*, inositol requiring; *spe*, spermidine requiring. CEN, centromere.

map of *N. crassa*, or approximately 600 kb. Despite the leaky phenotype of JP100, it was nevertheless severely counterselected in crosses. This is indicated in Table II by the ratio of JP100 to *spe⁺* isolates in the cross involving that allele.

Leaky Growth and Polyamine Synthesis

On agar medium, strains bearing the JP120 and LV105 mutations grow indefinitely without spermidine, although growth of the latter is weak. JP100-bearing strains also grow initially, but stop after a time, depending upon inoculum size. Small liquid cultures show the same pattern (Table III): JP100 and LV105 mutants display partial, but clear spermidine auxotrophy. The polyamine pools of these cultures reflect their genetic deficiencies: putrescine accumulates, and the spermidine pool is low (Table IV).

The leakiness of *spe-2* and *spe-3* mutants allows them to grow quite well in minimal exponential cultures inoculated with the standard, heavy inoculum (about 6×10^6 conidia per milliliter). However, comparison of spermidine in the inoculum (ca. 1 nmol per milliliter final culture volume) with that in the resulting mycelium showed that the JP100 strain synthesized no net spermidine as it grew to 2 mg dry weight per milliliter (data not shown). The LV105 mutant synthesized a small amount of spermidine, and the JP120 mutant even more. We conclude that the *spe-2* mutation (JP100) completely blocks sper-

TABLE III

Growth in Stationary Culture[a]

Strain	*spe* allele	Growth (mg dry wt/10 ml) in		
		MIN[b]	PUT	SPD
aga	*spe⁺*	25	26	26
spe-1	LV10	0	27	32
spe-2	JP100	0.8	0.8	26
spe-3	LV105	4.9	4.6	25
	JP120	27	26	28

[a] Strains were grown in 10 ml medium at 32°C.
[b] Media: MIN, no supplement; PUT, SPD, supplemented with 1 mM putrescine or spermidine, respectively.

TABLE IV

Enzyme Activities and Polyamine Contents of Mutants and Revertants

Locus	*spe* allele	Medium	Enzyme activities		SDC K_m (mM SAM)	Polyamines		
			ODC	SDC		PUT	SPD	SPM
			(U^a/mg protein)			(nmol/mg dry wt)		
aga	*spe*[+]	MIN[b]	35	42	0.04	1.2	17	0.26
		SPD	13	44		1.1	25	0.32
		ARG	2038	90		0.26	2.6	0.38
spe-1	LV10	MIN	<1	198		0	1.9	0
spe-2	JP100	MIN	371	<1		26	1.6	0
	JPR1[c]	MIN	ND[d]	20	0.3	64	7.2	0
	JPR2	MIN	ND	19	0.3	27	5.0	0
spe-3	LV105	MIN	1058	67	0.03	33	0.7	0
	JP120	MIN	455	73	0.04	30	4.4	0

[a] 1 U = 1 nmol product per hour.
[b] Media: Minimal (MIN), or with 1 mM spermidine (SPD) or 1 mM arginine (ARG) supplementation.
[c] JPR designates revertants of JP100 (*spe-2*).
[d] ND = not determined.

midine synthesis, whereas the *spe-3* mutations (LV105 and JP120) do not.

S-Adenosyl Methionine Decarboxylase Assays

SDC and ODC were assayed in *aga* mycelia grown in minimal medium or in medium containing 1 mM spermidine or 1 mM arginine. Spermidine represses ODC activity; arginine leads to extreme derepression of ODC by causing the strain to starve for polyamines, owing to feedback inhibition of ornithine synthesis. SDC specific activity varies only over a 2- to 3-fold range in these growth conditions (Table IV), compared to the 70- to 100-fold derepression of ODC activity. Similar tests of the *spe-1*, *spe-2*, and *spe-3* mutants, grown in minimal medium revealed that the *spe-2* strain (carrying JP100) entirely lacks SDC activity. Strains carrying mutations LV105 and JP120 (*spe-3*), or LV10 (*spe-1*), when grown in minimal medium, have SDC activities in the normal range (Table IV). As expected, *spe-2* and *spe-3* mutants grown in minimal medium have high ODC activity.

The difficulty of obtaining dcSAM, a substrate of spermidine synthase, precluded assay of spermidine synthase. The fact that LV105 and JP120 (*spe-3*) block spermidine synthesis from putrescine, yet complement and/or recombine with the SDC-deficient JP100, imply that the *spe-3* strains lack spermidine synthase.

JP100 Is a spe-2 Structural Gene Mutation

Revertants of JP100 were selected to see whether one might have an altered form of SDC. Genetic analyses of two revertants, JPR1 and JPR2, demonstrated that the revertant events were inseparable from the original JP100 mutation and were probably not extragenic suppressors. The SDC of the revertants had high K_m's for

SAM, and lower than normal activity (Table IV). The polyamine pools of the revertants were intermediate between mutant and wild type (Table IV). The data implicate *spe-2* as the SDC structural gene.

Polyamine Pools and Growth

As noted, JP100 grows exponentially in liquid minimal medium if the inoculum, which contains spermidine, is large. Curiously, the doubling time of such a culture during early log phase is similar to that of wild type (Table V). Using cultures grown in minimal medium, we compared mycelia of the JP100 mutant to those of wild type and an ODC-less *spe-1* mutant. Both mutants contained low spermidine pools, yet JP100 grows much more rapidly. Even later in log phase, the JP100 mutant doubles in 6.4 h, whereas *spe-1* barely grows at all. Comparable cultures of wild type continue to double in 2.8 h.

The JP100-carrying strain accumulates putrescine when starved for spermidine, whereas *spe-1* has little or

TABLE V

Polyamine Pools and Exponential Growth Rates in Minimal Medium

Locus (allele)	Polyamines			Doubling time[a] (h)
	PUT	SPD	SPM	
	(nmol/mg dry wt)			
aga (*spe*[+] control)	0.91	15	0.27	2.8
spe-1 (LV10)	0	1.9	0	7.2
spe-2 (JP100)	26	1.6	0	2.6

[a] Doubling times for *spe-1* and *spe-2* are for the initial exponential growth phase, before growth slows.
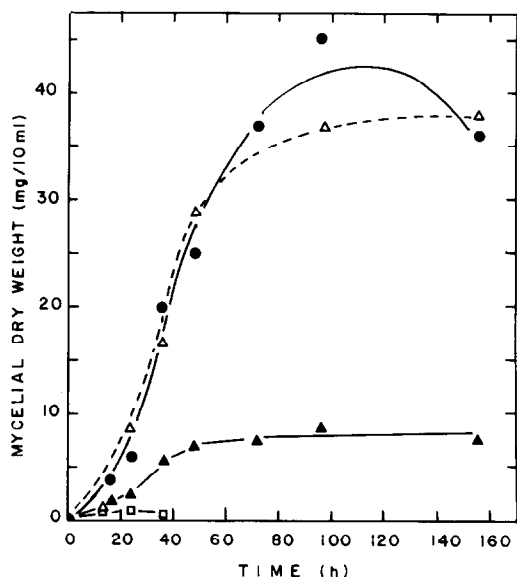
FIG. 3. Growth of Spe$^+$ and Spe$^-$ strains in stationary culture (10 ml). Closed circles: *aga* (*spe$^+$*) grown in minimal medium; open triangles: *spe-2* (JP100) grown with 1 mM spermidine; closed triangles: *spe-2* (JP100) grown in minimal medium; open squares, *spe-1* (LV10) grown in minimal medium.

no putrescine. This must in some way account for the difference in their growth in minimal medium. One might ask whether there is a minimum spermidine requirement for growth. Because of a clumped growth habit of polyamine-starved cells in exponential culture, we explored this question with lightly inoculated cultures of the JP100 mutant in small, stationary flasks of minimal medium. Entire cultures were harvested and analyzed at various times. The inocula were adjusted so that the strain carrying the JP100 mutation (*spe-2*) would cease growth due to polyamine limitation. The *spe-1* strain barely grew at all; after 36 h the culture started to die and its growth became unmeasurable (Fig. 3). The *spe-2* mutant grew in minimal medium for 2 days and plateaued at a low dry weight. When grown with 1 mM spermidine, this strain grew as well as a Spe$^+$ strain on minimal medium.

The polyamine pools of the *spe-1* (LV10) and *spe-2* (JP100) mutants showed the difference seen in exponential culture, although the absolute values were different. The *spe-1* strain has little putrescine, and one-fifth the normal amount of spermidine (3–4 nmol spermidine per milligram, dry weight). The *spe-2* strain had a large putrescine pool, and only 1/60th the normal amount of spermidine (0.3 nmol spermidine per milligram, dry weight) as it ceased growth. The data suggest that the large putrescine pool supports a substantial increase in weight until spermidine declines below a critical concentration.

DISCUSSION

Among the ca. 30 mutations in polyamine biosynthesis isolated in *N. crassa*, the great majority lie in the

ODC gene, *spe-1*. The new mutations described here define the other two main steps in the pathway, SDC [*spe-2* (JP100)] and spermidine synthase [*spe-3* (LV105 and JP120)]. The altered SDC activity in *spe-2* revertants strongly indicates that *spe-2* is the SDC structural gene. The proposed spermidine synthase deficiency of *spe-3* mutants is provisional, pending direct assay of the enzyme. The difficulty of isolating *spe-2* and *spe-3* mutants is caused at least in part by their leakiness, which militates against their selection. Similar difficulties were encountered in the first study of such mutants in *Saccharomyces cerevisiae* (17), and another study in yeast yielded *spe-2* mutants only after direct screening for SDC activity (18).

The genetic relationship of the three loci in *N. crassa* is peculiar: all three lie in the 21-cM region of chromosome V. We attach no significance to this presently, but related genes of other pathways are occasionally linked loosely in suggestive ways. Conceivably the coordinated activity of these clustered genes is served by their common chromosomal domain, but translocations of parts of other such clusters impart no obvious impairment to the phenotype [e.g., (19)].

We infer from the limited regulatory information given here that ODC specific activity is controlled over a far greater range than that of SDC. This suggests that ODC is the more important enzyme in controlling the rate of spermidine synthesis. Neither ODC nor SDC is feedback inhibited by spermidine. Polyamines control ODC activity by a combination of effects on enzyme synthesis and enzyme turnover (20), and SDC activity is controlled by the availability of putrescine, an obligatory cofactor (12). Thus by variation of ODC activity, SDC is secondarily controlled, and the concentrations of the two substrates of spermidine synthesis are coordinated.

The relative importance of putrescine and spermidine have been debated in a number of organisms, once the polyamines themselves were identified as essential (21). The new mutants described here show that spermidine is an essential metabolite in *N. crassa*, as it is in yeast (17). However, a comparison of *spe-1* and the *spe-2/3* mutants shows that excess putrescine radically reduces the spermidine requirement of *N. crassa*. One may offer two interpretations, not mutually exclusive, of this observation. (i) A high concentration of the divalent putrescine may substitute for the trivalent spermidine in many of the roles of the latter. Studies of a number of macromolecular interactions *in vitro* demonstrate some interchangeability of the polyamines with one another (and with Mg^{2+}), particularly where their cationic character is important. (ii) A high concentration of putrescine may render spermidine more effective in its essential role(s). Our previous work demonstrated binding of most of the cellular spermidine of *N. crassa* to intracellular constituents (5). As a consequence, upon starvation for spermidine, *N. crassa* mounts an extreme regulatory

response before the cellular spermidine pool is significantly reduced (10). Thus if a high level of putrescine displaced residual spermidine to a diffusible state, the organism could withstand a greater degree of spermidine starvation. We cannot judge the relative importance of these possibilities with our data, but the process having the most stringent spermidine requirement might be identifiable by use of *spe-2* or *spe-3* mutants.

In yeast, the minimal spermidine requirements of *spe-1* and *spe-2* mutants are difficult to judge from the published data, owing to the slight leakiness of the mutants tested, and to a lesser putrescine accumulation in cells of the latter grown in spermidine-free medium (17). Nevertheless, yeast seems to have a lower polyamine requirement than *N. crassa*, because growth continues indefinitely (though slowly) after spermidine pools are reduced to less than 1% of normal (17, 18).

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